

A Signal Transduction Pathway from TGF- β 1 to SKP2 via Akt1 and c-Myc and its Correlation with Progression in Human Melanoma

Xuan Qu^{1,7}, Liangliang Shen^{2,7}, Yan Zheng³, Yang Cui¹, Zhihui Feng⁴, Feng Liu^{5,6} and Jiankang Liu¹

Both SKP2 (S-phase kinase-associated protein 2) and transforming growth factor- β 1 (TGF- β 1) play important roles in cancer metastasis through different mechanisms: TGF- β 1 via induction of epithelial–mesenchymal transition (EMT) and SKP2 via downregulating p27^{kip1}. Recent studies indicated that c-Myc and Akt1 were active players in metastasis. In this study we demonstrated a crosstalk between these pathways. Specifically, we found that TGF- β 1 treatment increased SKP2 expression accompanied with increased phosphorylation of Akt1 and c-Myc protein accumulation during EMT. We demonstrated that Akt1 was required for TGF- β 1-mediated SKP2 upregulation and that c-Myc transcription factor specifically bound to the promoter of SKP2 for its enhanced transcription. Analysis of 25 samples of normal human skin, nevi, and melanomas revealed a positive correlation between c-Myc and SKP2 accumulation. Furthermore, accumulation of SKP2 and c-Myc proteins was significantly higher in metastatic melanoma samples as compared with that in primary melanomas, which again was higher than that in normal skin or nevi. In summary, our results integrated TGF- β 1 signals to SKP2 via Akt1 and c-Myc during EMT, and provided, to our knowledge, a previously unreported mechanistic molecular event for TGF- β 1-induced metastasis in human melanoma.

Journal of Investigative Dermatology (2014) **134**, 159–167; doi:10.1038/jid.2013.281; published online 18 July 2013

INTRODUCTION

Melanoma represents \sim 4% of human skin cancers, yet accounts for nearly 80% of deaths from skin tumors (Houghton and Polsky, 2002). Its high mortality rate is mainly a result of the propensity to metastasis. Further understanding of the molecular mechanisms that enable melanoma invasion is urgently needed.

¹Institute of Mitochondrial Biology and Medicine, The Key Laboratory of Biomedical Information Engineering of Ministry of Education, Xi'an Jiaotong University School of Life Science and Technology, Xi'an, China; ²Department of Biochemistry and Molecular Biology, The State Key Laboratory of Cancer Biology, The Fourth Military Medical University, Xi'an, China; ³Department of Dermatology, The Second Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China; ⁴Center for Mitochondrial Biology and Medicine, Frontier Institute of Science and Technology, Xi'an Jiaotong University, Xi'an, China; ⁵Department of Medicine, University of California Irvine Medical School, Irvine, California, USA and ⁶Chao Family Comprehensive Cancer Center, University of California Irvine Medical School, Irvine, California, USA

⁷These authors contributed equally to this work.

Correspondence: Yan Zheng, Department of Dermatology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China, 710004, China. E-mail: zenyan66@126.com or Feng Liu, Department of Medicine, Chao Family Comprehensive Cancer Center, University of California Irvine School of Medicine, 839 Health Science Road, B200 Sprague Hall, Irvine, California 92697, USA. E-mail: liufe@uci.edu

Abbreviations: Akt, acutely transforming retrovirus AKT8 in rodent T cell lymphoma; ChIP, chromatin immunoprecipitation; E-cadherin, epithelial cadherin; EMT, epithelial–mesenchymal transition; N-cadherin, neural cadherin; NHM, normal human melanocyte; PI3K, phosphoinositide-3-kinase; shRNA, short hairpin RNA; siRNA, small interfering RNA; SKP2, S-phase kinase-associated protein 2; TGF- β , transforming growth factor- β

Received 1 May 2012; revised 12 October 2012; accepted 29 October 2012; accepted article preview online 21 June 2013; published online 18 July 2013

The phosphoinositide-3-kinase (PI3K)/Akt pathway controls many fundamental processes of cancer cell biology including cell proliferation, differentiation, and apoptosis. The transforming growth factor- β (TGF- β) can rapidly activate PI3K via phosphorylation of Akt (Bakin *et al.*, 2000), which appears to be independent of Smad2/3 activation (Wilkes *et al.*, 2005). The TGF- β /PI3K/Akt pathway plays a central role in epithelial–mesenchymal transition (EMT) (Larue and Bellacosa, 2005), which facilitates cell migration and invasion by changing cell morphology and extracellular matrix components. During EMT, cells lose expression of epithelial markers such as epithelial cadherin (E-cadherin) and increase mesenchymal markers such as neural cadherin (N-cadherin), vimentin, Snail, Twist, and fibronectin (Zeisberg and Neilson, 2009). Emerging lines of evidence suggest that tumor metastasis may be dependent on the acquisition of EMT features by primary cancer cells (Zavadil and Bottinger, 2005; Acloque *et al.*, 2009; Kalluri and Weinberg, 2009).

In addition to the PI3K/Akt pathway, c-Myc also plays a role during TGF- β -induced EMT (Smith *et al.*, 2009). It has been demonstrated that high TGF- β levels are often associated with melanoma progression (Javelaud *et al.*, 2008), and so does the Akt1, c-Myc, and SKP2 (S-phase kinase-associated protein 2) levels (Grover *et al.*, 1996; Schlagbauer-Wadl *et al.*, 1999; Dhawan *et al.*, 2002; Li *et al.*, 2004b; Robertson, 2005; Govindarajan *et al.*, 2007; Rose *et al.*, 2011). However, it is not clear how these signals are interacted and integrated in melanoma metastasis.

SKP2 is the substrate recognition subunit of SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex (Nakayama and Nakayama, 2005). Aberrant SKP2 expression plays an active role in tumorigenesis owing to its central role in degradation of a number of cyclin-dependent kinase inhibitors including p27^{kip1}, p21^{cip1}, and p57 (Tsvetkov *et al.*, 1999; Bornstein *et al.*, 2003; Kamura *et al.*, 2003). SKP2 was overexpressed in melanoma and its levels were correlated with metastasis (Yokoi *et al.*, 2004; Li *et al.*, 2004a; Rose *et al.*, 2011). SKP2 regulates c-Myc protein stability and activity at both transcriptional and post-translational levels (Kim *et al.*, 2003). Whether and how SKP2 is regulated during TGF- β -induced EMT remains to be elucidated.

RESULTS

TGF- β 1-induced EMT and SKP2 expression in melanoma cells

In order to study the TGF- β 1-mediated EMT signal cascade and whether it involves SKP2, the A375 and SK-MEL-28 melanoma cell lines were treated with TGF- β 1 and EMT

was examined at various time points. EMT was measured by both cell morphology and the expression of biomarkers including E-cadherin, N-cadherin, fibronectin, and Snail. A375 cells exhibited cobblestone-like morphology under normal culture condition, with or without serum (Figure 1a, and data not shown). TGF- β 1 was added to serum-free media at concentrations of 5 or 10 ng ml⁻¹. The cells began to acquire an elongated mesenchymal-like phenotype after 24 hours (Figure 1a), which was enhanced 48 hours after treatment, with most cells displaying an elongated and fibroblast-like morphology (Figure 1a). Moreover, the cells treated with TGF- β 1 for 2 days acquired a mesenchymal mRNA expression profile, as evidenced by increased accumulation of the mRNAs encoding N-cadherin, Snail, and fibronectin and decreased accumulation of E-cadherin mRNA (Figure 1b). Consistently, protein-level changes of EMT markers were also observed in A375 and SK-MEL-28 cells (Figure 1c). Upregulation of SKP2 is recognized as an important mechanism for tumorigenesis and metastasis in

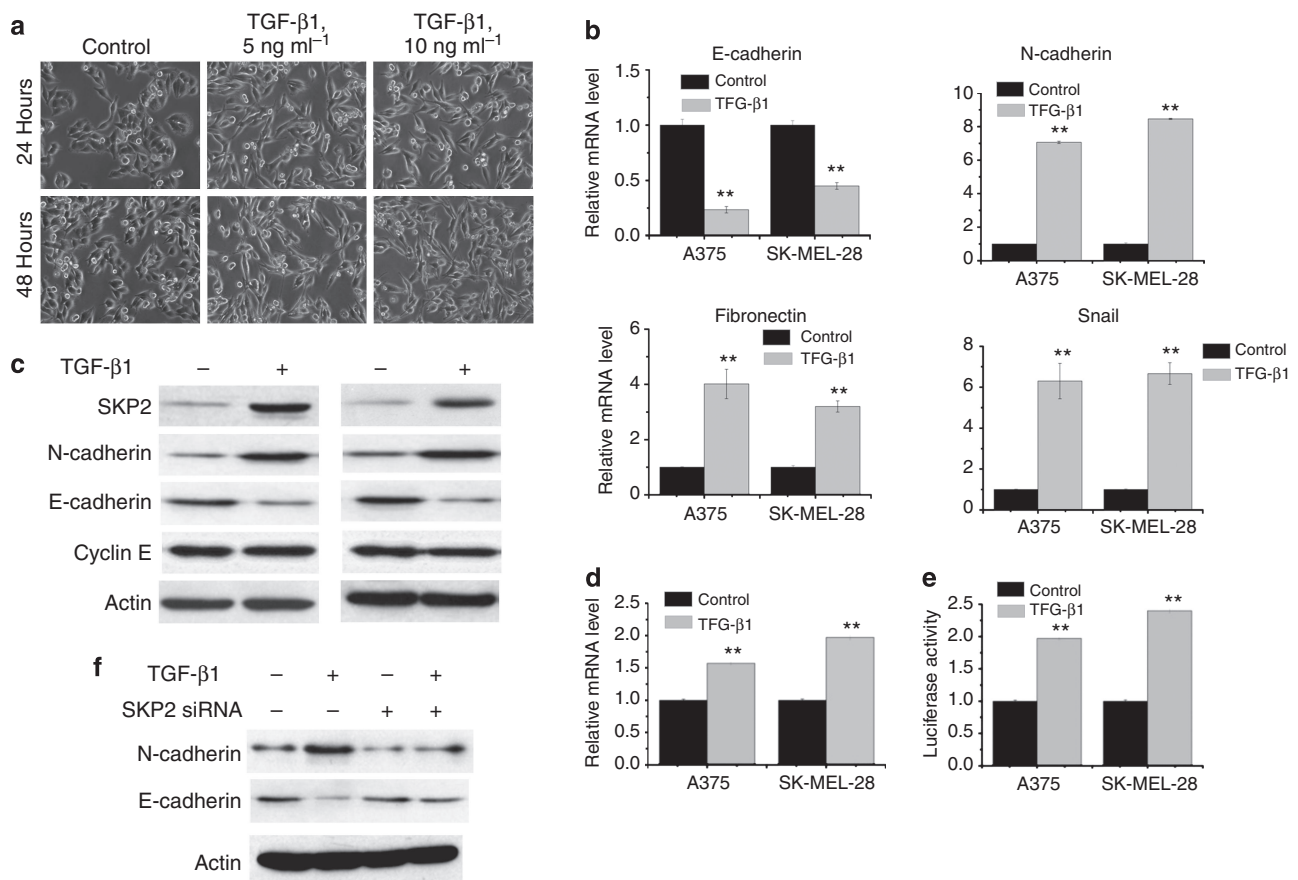


Figure 1. Transforming growth factor- β 1 (TGF- β 1)-induced epithelial-mesenchymal transition (EMT) and S-phase kinase-associated protein 2 (SKP2) expression in melanoma cells. (a) Morphological changes of A375 cells treated with TGF- β 1. (b) Quantitative reverse-transcriptase-PCR (qRT-PCR) quantification of epithelial cadherin (E-cadherin), neural cadherin (N-cadherin), fibronectin, and Snail in A375 and SK-MEL-28 cells treated with vehicle or TGF- β 1 (10 ng ml⁻¹) for 2 days. The control was defined as 1.0. Data shown are means \pm SD of triplicates and represent three independent experiments (same for all the following experiments). (c) Western blot analysis of SKP2, N-cadherin, E-cadherin, and cyclin E in A375 and SK-MEL-28 cells treated with TGF- β 1 (10 ng ml⁻¹, 2 days). (d) qRT-PCR results showing SKP2 transcription levels in A375 and SK-MEL-28 cells treated with vehicle or TGF- β 1 (10 ng ml⁻¹, 2 days). (e) Dual luciferase assay results showing the SKP2 promoter activity increases after TGF- β 1 treatment (10 ng ml⁻¹, 1 day). (f) Western blot analysis of N-cadherin and E-cadherin in A375 cells treated with TGF- β 1 or SKP2 small interfering RNA (siRNA). ** P < 0.01, Student's t -test.

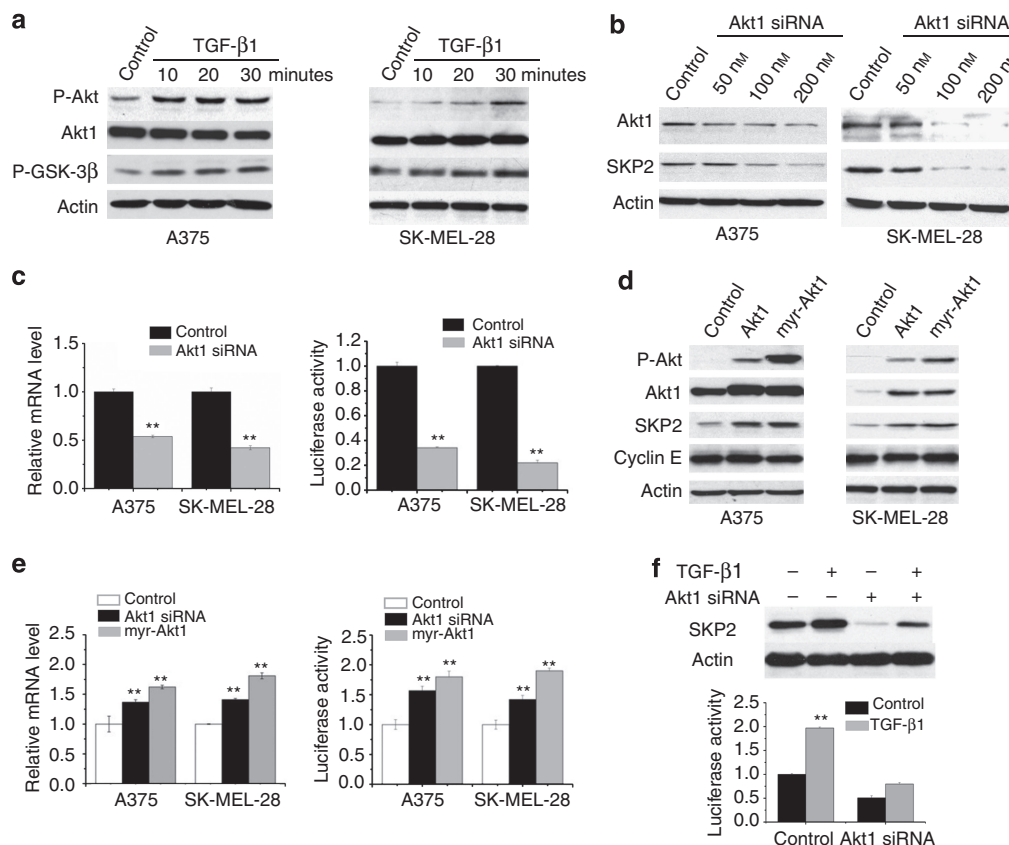


Figure 2. Transforming growth factor- β 1 (TGF- β 1) induces S-phase kinase-associated protein 2 (SKP2) activation via Akt1. (a) Western blot analysis of time-dependent activation of Akt (acutely transforming retrovirus AKT8 in rodent T cell lymphoma) in TGF- β 1-treated A375 and SK-MEL-28 cells (10 ng ml $^{-1}$). P-GSK-3 β , phosphorylated glycogen synthase kinase-3 β . (b) Western blot analysis of knockdown of Akt1 in A375 and SK-MEL-28 cells. (c) SKP2 mRNA and promoter activity was decreased by Akt1 small interfering RNA (siRNA). (d) Overexpression of the wild type or activating form of Akt1 (myr-Akt1) increased SKP2 protein. (e) SKP2 mRNA accumulation, as well as SKP2 promoter activity, was induced by Akt1. (f) AKT1 knockdown decreased SKP2 expression at transcriptional and protein levels. Top western blot analysis of SKP2 in A375 transfected with the Akt1 siRNA or control vector and treated with vehicle or TGF- β 1 (10 ng ml $^{-1}$, 48 hours). Bottom, SKP2 promoter reporter assay in cells described in the top panel. ** $P < 0.01$, Student's t -test.

melanoma (Katagiri *et al.*, 2006; Rose *et al.*, 2011), but its role in EMT has not been previously reported. In our experiment, at 2 days after TGF- β 1 stimulation, SKP2 protein accumulation increased in A375 and SK-MEL-28 cells (Figure 1c). Meanwhile, cyclin E, an early S-phase marker, was not induced (Figure 1c). The endogenous SKP2 mRNA level increased approximately 1.5- to 2-fold ($P < 0.01$; t -test) after TGF- β 1 stimulation in both cell lines (Figure 1d), suggesting that TGF- β 1 induced SKP2 expression at the transcriptional level, and was not influenced by cell cycle in this case. To further confirm our finding, a human SKP2 promoter-luciferase reporter plasmid (pGL4-SKP2-1.2) was transfected into cells together with an internal control pRL-SV40 plasmid. Dual luciferase reporter assay indicated that SKP2 promoter activity increased significantly upon TGF- β 1 stimulation ($P < 0.01$; t -test; Figure 1e). Furthermore, SKP2 knockdown by small interfering RNA (siRNA) dramatically inhibited the changes of E-cadherin and N-cadherin after incubation with TGF- β 1 (Figure 1f). Taken together, the above results suggest that SKP2 mediates TGF- β -induced EMT in melanoma cells.

TGF- β 1 induces SKP2 expression via Akt1

Next, we examined the mechanism by which SKP2 was upregulated by TGF- β 1 signaling. Activation of the Akt pathway is known to function as an adaptive signaling pathway triggered by TGF- β 1 (Bakin *et al.*, 2000). Therefore, we investigated whether Akt1 was involved in the cellular responses to TGF- β 1. Western blot analysis revealed a time-dependent increase in Akt phosphorylation at serine 473 after TGF- β 1 treatment. Meanwhile, glycogen synthase kinase-3 β , a substrate of PI3K/Akt pathway (Cross *et al.*, 1995), was also phosphorylated time-dependently via TGF- β 1 treatment (Figure 2a).

It was previously demonstrated that Akt1 regulated SKP2 mRNA and protein abundance in pancreatic ductal adenocarcinoma cells (Reichert *et al.*, 2007). To examine the role of Akt1 in SKP2 expression in melanoma cells, endogenous Akt1 was knocked down by siRNA. The Akt1 protein decreased to undetectable levels, which led to a concomitant reduction of SKP2 protein accumulation (Figure 2b). Consistently, Akt1 siRNA (100 nM) reduced SKP2 mRNA by 50–60% in A375 and SK-MEL-28 cells ($P < 0.01$; t -test; Figure 2c). Furthermore, Akt1 siRNA also led to a 60–80% reduction of SKP2 promoter

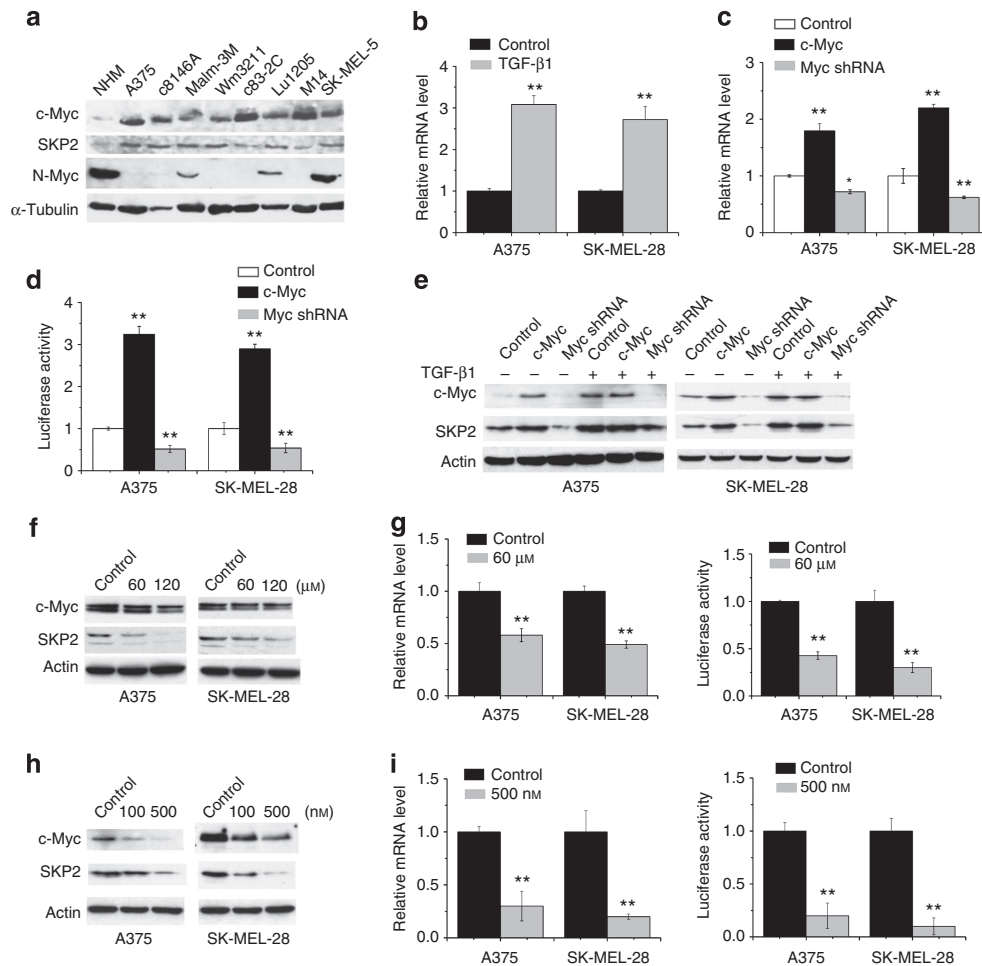


Figure 3. c-Myc positively regulates S-phase kinase-associated protein 2 (SKP2) transcription in response to transforming growth factor- β 1 (TGF- β 1) stimulation.

(a) Protein levels of c-Myc, N-Myc, and SKP2 in normal human melanocytes (NHMs) and melanoma cell lines. (b) c-Myc mRNA was induced by TGF- β 1 in A375 and SK-MEL-28 cells. (c) c-Myc overexpression or knockdown with/without TGF led to SKP2 mRNA level changes in A375 and SK-MEL-28 cells. shRNA, short hairpin RNA. (d) c-Myc overexpression or knockdown led to SKP2 promoter activity changes in A375 and SK-MEL-28 cells. (e) c-Myc overexpression or knockdown correlated with SKP2 protein levels in A375 and SK-MEL-28 cells. (f, g) c-Myc inhibitor F-10048 (60–120 μ M) decreased SKP2 protein and mRNA accumulation, as well as promoter activity. (h, i) c-Myc inhibitor JQ-1 (100–500 nM) decreased SKP2 protein and mRNA accumulation as well as promoter activity. * $P < 0.05$, Student's *t*-test. ** $P < 0.01$, Student's *t*-test.

activity as analyzed by luciferase reporter assays in both cell lines ($P < 0.01$; *t*-test; Figure 2c).

On the other hand, ectopic expression of Akt1 (pcDNA3.1-Akt1) or a constitutively active Akt1 that contained a myristoylation sequence (pcDNA3.1-myr-Akt1) (Shen *et al.*, 2010) dramatically increased SKP2 protein levels (Figure 2d). Ectopic expression of Akt1 in melanoma cells also moderately but consistently increased the accumulation of SKP2 mRNA and promoter activity ($P < 0.01$; *t*-test; Figure 2e). Next, we examined the effect of Akt1 on TGF- β 1-induced transcriptional activation of SKP2. As shown in Figure 2f, Akt1 siRNA inhibited the TGF- β 1-mediated induction of both SKP2 protein level and promoter activity.

c-Myc positively regulates SKP2 transcription in response to TGF- β 1 stimulation

We next sought to identify the transcription factor responsible for SKP2 gene induction in response to TGF- β 1 stimulation. Using TESS (Transcription Element Search System) program

(<http://www.cbil.upenn.edu/cgi-bin/tess/tess>), several E-box cis-elements (CACGTG, Myc-binding sites) were found in the SKP2 promoter. A previous study has shown that c-Myc activates and binds to the SKP2 promoter in leukemia cells (Bretones *et al.*, 2011). Whether TGF- β 1-mediated SKP2 activation is through c-Myc is still unknown.

Expression of SKP2 in normal human melanocytes (NHMs) and melanoma cell lines was examined by western blot. Low level of SKP2 protein was detected in NHMs; higher level of SKP2 protein was detected in all melanoma cell lines examined (Figure 3a). Consistent with the published data, c-Myc protein accumulation was also elevated in all melanoma cell lines as compared with NHMs. Interestingly, N-Myc was expressed in NHMs but lost in some melanoma cell lines including A375, c8146A, Wm3211, c83-2C, and M14 (Figure 3a). These results suggest that c-Myc but not N-Myc expression may be correlated with SKP2 expression in human melanoma cell lines. Therefore, we further investigate the relationship between c-Myc and SKP2.

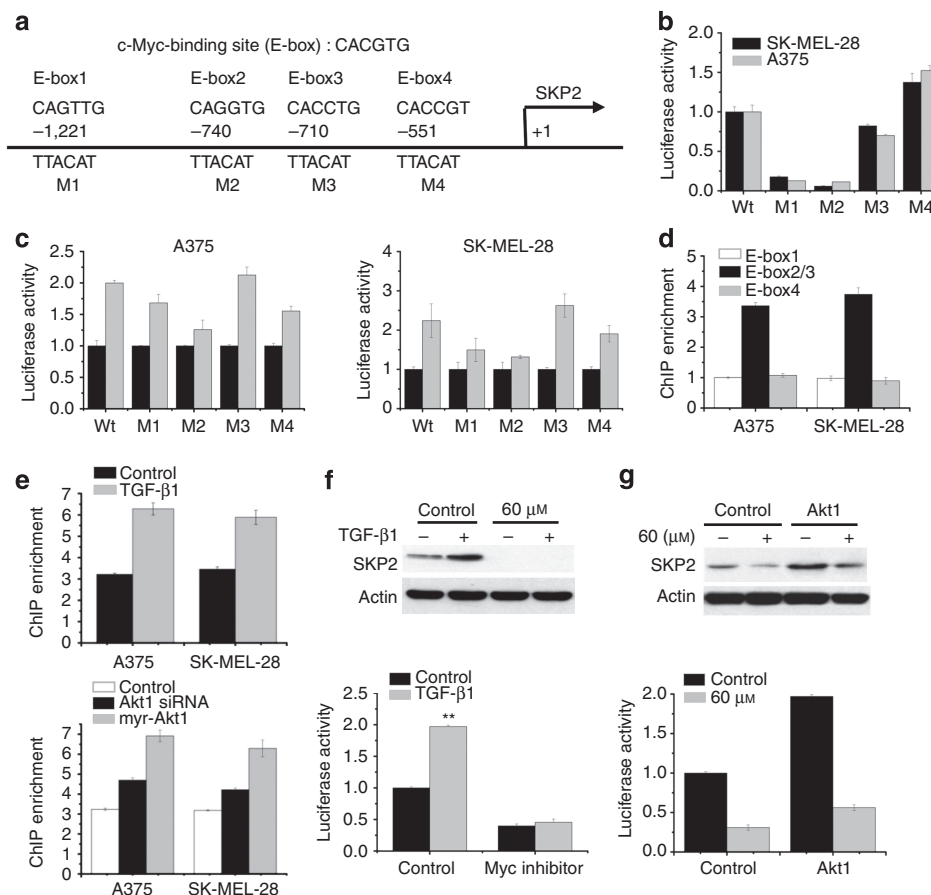


Figure 4. c-Myc binds to *S*-phase kinase-associated protein 2 (SKP2) promoter via E2 box and mediates transforming growth factor- β 1 (TGF- β 1)-induced SKP2 expression. (a) Promoter region of the SKP2 gene. Four potential c-Myc-binding sites are shown. (b) Relative reporter activity of PGL4-SKP2, M1, M2, M3, and M4 in A375 and SK-MEL-28 cells. (c) c-Myc inducibility of the SKP2 promoters in SK-MEL-28 and A375 cells. (d) Chromatin immunoprecipitation (ChIP) assay showing c-Myc binds to E-box2/3 of SKP2 promoter in SK-MEL-28 and A375 cells. (e) TGF-induced (upper panel) and Akt1-induced (lower panel) c-Myc binding to SKP2 promoter in A375 and SK-MEL-28 cells. (f) Western blot analysis of SKP2 protein level (upper panel) and promoter activity (lower panel) in A375 cells treated with c-Myc inhibitor (60 μ M) and TGF (10 ng ml $^{-1}$) for 24 hours. (g) Western blot analysis of SKP2 protein level (upper panel) and promoter activity (lower panel) in A375 cells transfected with Akt1 vector, followed by c-Myc inhibitor (60 μ M, 24 hours). ** $P < 0.01$, Student's t -test.

To dissect this signal pathway, quantitative reverse-transcriptase-PCR analysis indicated that 2 days after TGF- β 1 stimulation, the level of c-Myc mRNA expression was increased ~ 3 -fold ($P < 0.01$; t -test; Figure 3b), indicating that c-Myc was also upregulated at the transcriptional level by TGF- β 1. To investigate the importance of the presence of c-Myc in conferring the transcription inducibility of SKP2 by TGF- β , we transfected the empty vector, c-Myc overexpression construct, and c-Myc short hairpin RNA (shRNA) construct into A375 or SK-MEL-28 cell lines, respectively. SKP2 mRNA levels and promoter activity were increased by c-Myc overexpression ($P < 0.01$; t -test), whereas the c-Myc shRNA construct decreased SKP2 mRNA and promoter activity significantly ($P < 0.01$; t -test; Figure 3c and d). Moreover, as shown in Figure 3e, the expressions of c-Myc and SKP2 were remarkably induced in the presence of TGF- β 1. However, TGF- β 1-induced SKP2 expression was not completely abolished when c-Myc was knocked down (comparing the two "Myc shRNA" lanes without or with TGF- β 1 treatment in each cell line).

Consistent with the above results, when c-Myc inhibitor 10058-F4 (60 or 120 μ M) (Huang *et al.*, 2006; Lin *et al.*, 2007) was used to treat A375 or SK-MEL-28 cells, SKP2 protein level was reduced after 24 hours in a dose-dependent manner (Figure 3f). At the transcriptional level, 10058-F4 (60 μ M) effectively decreased SKP2 mRNA accumulation and promoter activity in both melanoma cells (Figure 3g). No visible cell death was observed in 10058-F4-treated cells (data not shown). Similar results were also found with JQ1 treatment at 100 and 500 nM, which is another effective c-Myc inhibitor (Figure 3h and i).

c-Myc binds to SKP2 promoter via E2 box and mediates TGF- β 1-induced SKP2 expression

Four potential c-Myc-binding sites (E-box: 5'-CACGTG-3') were located within the 1.2 kb of the human SKP2 promoter (Figure 4a). To determine which E-box is responsible for c-Myc binding, we generated site-directed mutations on all four potential c-Myc-binding sites named M1, M2, M3, and M4, representing mutations on E-box1/2/3/4, respectively.

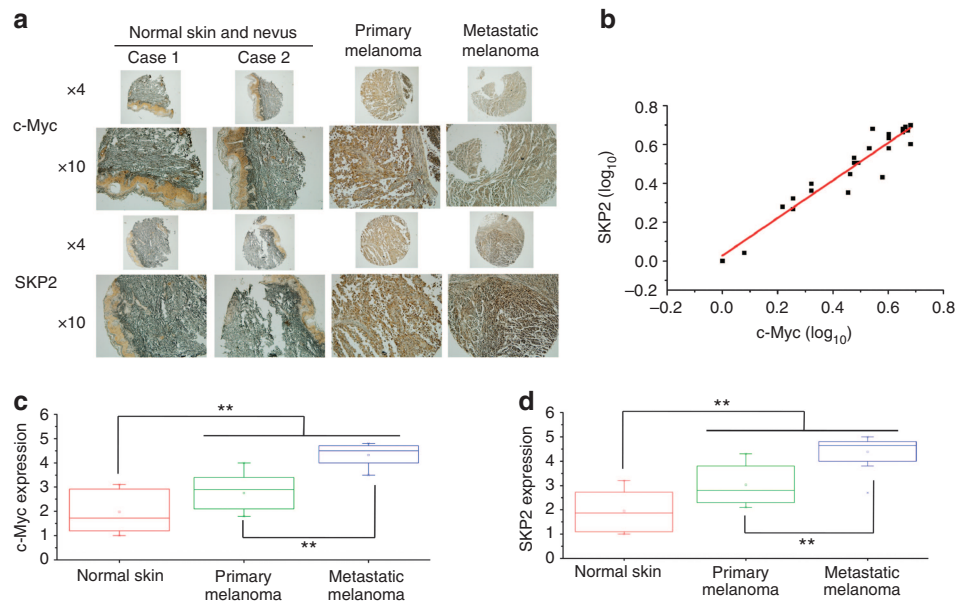


Figure 5. Correlation of c-Myc and S-phase kinase-associated protein 2 (SKP2) levels in melanoma tumor samples. (a) Immunohistochemical staining of c-Myc and SKP2 in human normal skin, nevus, and melanoma samples. SKP2 is enriched in melanoma and correlates with c-Myc expression. Original magnification: $\times 4$, $\times 10$, or $\times 20$. (b) Correlation between c-Myc and SKP2 expression in 25 human tissues, with linear regression lines and Pearson's correlation significance ($P < 0.001$, analysis of variance (ANOVA) test). (c, d) Relative expression levels of c-Myc and SKP2 in normal skin, nevus, and melanoma. * $P < 0.05$, ** $P < 0.01$, unpaired Student's *t*-test.

The human wild-type *SKP2* promoter and these four E-box mutations were transfected into A375 and SK-MEL-28 cells, respectively. As shown in Figure 4b, mutagenesis of E-box4 (M4) had no effect on the activity of the *SKP2* promoter. Mutagenesis of E-box3 (M3) decreased the luciferase activity of *SKP2* by at least 30%. In addition, M1 and M2 mutations decreased the *SKP2* luciferase activity by nearly 90%. These results indicated that the binding sites of E-box1 and E-box2 are critical for the *SKP2* promoter activity.

Next, the response of pGL4-*SKP2* mutations to c-Myc overexpression was further investigated. The *SKP2* wild-type promoter activity was activated approximately 2- to 3-fold by c-Myc overexpression in A375 cells and SK-MEL-28 cells, respectively (Figure 4c), whereas mutagenesis of E-box1 and E-box4 slightly reduced the inducibility by c-Myc; mutation of E-box3 did not significantly affect the inducibility by c-Myc in both cell lines. Mutation of E-box2 dramatically decreased the activation of *SKP2* promoter by c-Myc, suggesting that the E-box2 may be the major site for c-Myc to regulate *SKP2* transcription (Figure 4c). To further confirm whether c-Myc binds to the promoter directly, we carried out chromatin immunoprecipitation (ChIP) assay in SK-MEL-28 and A375 cells. Figure 4d shows that the primers corresponding to E-box2/3 regions yielded significant amounts of product; in contrast, the E-box1 and E-box4 region yielded no specific signal (Figure 4d). Together, these results indicated that the E-box2 in the *SKP2* promoter is responsible for mediating c-Myc-induced *SKP2* activation.

To further dissect the induction of *SKP2* in response to upstream signals, melanoma cells were treated with TGF- β 1 or

transfected with Akt1 constructs. ChIP experiments indicated that the binding of c-Myc to *SKP2* promoter was dramatically increased by TGF- β 1 treatment or Akt1 overexpression (Figure 4e). Moreover, in the presence of c-Myc inhibitor 51008-F4, TGF- β 1-induced or Akt1 overexpression-induced *SKP2* expression was partly abolished at both transcriptional and protein levels in melanoma cells (Figure 4f and g). These data suggest that c-Myc was required in mediating TGF- β 1-activated *SKP2* expression.

Correlation of c-Myc and SKP2 levels in melanoma tumor samples

To measure whether there is a clinical correlation between c-Myc and SKP2 in human tissue samples, and whether these proteins are correlated with melanoma progression, we performed immunohistochemical analysis to assess the expression levels of SKP2 and c-Myc in 2 human normal skin samples, 6 benign nevi, 7 primary melanoma, and 10 metastatic melanoma samples. As shown in Figure 5a, human normal skin tissue and nevi did not exhibit any positive staining for SKP2 or c-Myc. In contrast, the expression levels of both c-Myc and SKP2 were upregulated in primary melanoma samples, and even more so in metastatic melanomas. Furthermore, an association study showed that c-Myc expression positively correlated with SKP2 expression in these tissue samples ($P < 0.001$; analysis of variance test; Figure 5b). The average fold change of c-Myc expression in metastatic melanoma was significantly higher than in unpaired primary melanoma (4.32 vs. 2.75; $P = 0.00012$; unpaired Student's *t*-test; Figure 5c). Similarly, the average fold change of SKP2 expression in metastatic melanoma was significantly higher

than that in unpaired primary melanoma (4.31 vs. 3.02; $P=0.0025$; unpaired Student's *t*-test; Figure 5d). The demonstration of SKP2 expression in distal metastases supports our conclusion that SKP2 expression in melanoma correlates with disease progression. These *in vivo* findings are consistent with previous *in vitro* observations that c-Myc transcription factor can induce SKP2 expression.

DISCUSSION

SKP2 is a critical regulator of cell cycle progression that targets several cyclin-dependent kinase inhibitors for degradation. Given the central role of SKP2 cell cycle regulation, aberrant activation of SKP2 gene expression has been detected in a large number of human cancers, and high level of SKP2 is strongly correlated with tumor progression and poor prognosis. Thus, identification of the regulatory mechanisms leading to increased SKP2 expression may offer an insight into the control of tumor cell proliferation and progression.

In this study, we found that SKP2 expression was increased by TGF- β 1 treatment in melanoma cells, accompanied by the changes in EMT markers and cell morphology. We further found that PI3K/Akt signaling played a role in TGF- β 1-induced SKP2 expression. The PI3K/Akt pathway regulates many fundamental processes of cancer cell biology, and also functions as an adaptive signaling pathway triggered by TGF- β 1. Here our study showed that knockdown of the endogenous Akt1 in melanoma cells repressed SKP2 expression at the transcriptional level. On the other hand, enforced expression of Akt1 increased SKP2 mRNA and protein expression, as well as SKP2 promoter activity. Moreover, knockdown of endogenous Akt1 is sufficient to eliminate the TGF- β 1-induced SKP2 expression in melanoma cells. Therefore, the TGF- β 1-mediated SKP2 gene upregulation is partly through Akt1.

However, enforced expression of Akt1 is not sufficient in itself to explain the strong induction of SKP2 by TGF- β 1 in melanoma cells. Compared with this induction, the induction of SKP2 by enforced Akt1 expression is still weak. Thus, in its natural setting, the SKP2 promoter must receive further TGF- β 1 inputs for activation. In our experiment, four E-boxes were found in SKP2 core promoter region that may be induced by TGF- β 1. Several lines of evidence indicated that c-Myc was required in the induction of SKP2 expression by TGF- β 1 in melanoma cells: (1) transfection of c-Myc activated the SKP2 expression; (2) c-Myc inhibitor represses the SKP2 expression; (3) TGF- β 1 induced c-Myc expression at the transcriptional level; (4) knockdown of c-Myc by siRNA or its inhibitor abolished the induction of SKP2 by TGF- β 1; and (5) c-Myc directly binds to SKP2 core promoter. The significance of the binding sites was further demonstrated by the fact that mutation of E-box2 reduced the SKP2 promoter activity, as well as inducibility by TGF- β 1. Moreover, ChIP assay demonstrated that c-Myc bound to specific regions of SKP2 promoter. These experiments may explain, on one hand, the requirement of c-Myc for TGF- β 1-mediated activation of SKP2 and, on the other, the ability of TGF- β 1 to provide a second input for SKP2 activation in addition to Akt1 signaling pathway.

Immunohistochemical staining of melanoma samples for c-Myc and SKP2 showed a correlation of protein accumulation between these two proteins, which is also positively correlated with melanoma progression. Our analysis of 25 tumor samples demonstrated that c-Myc-mediated activation likely serves as an important guardian for overexpression of SKP2 *in vivo*. It should be noted that, as is often observed among clinical samples, no 1:1 correlation between c-Myc and SKP2 expression in tissues was observed. Thus, in addition to c-Myc, other factors may also contribute to TGF- β 1-induced SKP2 expression and melanoma progression. This was further supported by our *in vitro* study in cell lines where c-Myc knockdown did not completely abolish the TGF- β 1-induced SKP2 protein accumulation (Figure 3e). As an important regulator for cell cycle progression and tumor invasion, SKP2 is regulated by many genes via transcriptional and post-translational processes, including E2F/Rb pathway, mTOR (mammalian target of rapamycin) pathway, glypican-1 (GPC1), Stat3 (signal transducer and activator of transcription 3), and p300 (Assoian and Yung, 2008; Inuzuka *et al.*, 2012; Qiao *et al.*, 2012; Totary-Jain *et al.*, 2012). In melanoma, SKP2 level is also regulated by the BRAF pathway (Bhatt *et al.*, 2007). Further studies are warranted to understand the complexity of SKP2 regulation and function in melanoma.

Taking together our results and published data, regulation of SKP2 in the TGF- β 1-induced melanoma EMT integrated the PI3K/Akt1 and c-Myc oncogenic pathways in melanoma progression. SKP2 may be a critical effector in TGF- β 1-induced melanoma EMT process, and hence may serve as a good therapeutic target.

MATERIALS AND METHODS

Cell culture and reagents

Human melanoma cell lines (A375 and SK-MEL-28) were cultured in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. c-Myc inhibitors (10058-F4 and JQ-1) were purchased from Sigma (St Louis, MO) and Cayman Chemical (Ann Arbor, MI) separately. TGF- β 1 (240-B) was purchased from R&D Systems (Minneapolis, MN). Human skin and melanoma samples were collected with the written consent of patients; the experiment was approved by the Internal Review Board of Xi'an Jiao Tong University and adhered to the Declaration of Helsinki Principles.

Plasmid constructs and transfection

The human SKP2 promoter was subcloned from a previously published construct (Tang *et al.*, 2009) with the following steps: an *EcoRI/PstI* fragment from the published construct was first subcloned into pBSK to create pBSK-SKP2-1.2. A *KpnI/BamHI* fragment from pBSK-SKP2-1.2 was then released and ligated to pGL4.10 (*KpnI/BglII*), resulting in pGL4-SKP2-1.2. The pMXS-hc-Myc was purchased from Addgene.org (Cambridge, MA), and it originated from Dr Shinya Yamanaka's laboratory (Takahashi *et al.*, 2007). The c-Myc shRNA was also from Addgene.com (Popov *et al.*, 2007). Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

siRNA

The siRNAs (200 nm) against Akt1 (Irie *et al.*, 2005) and negative control (GenePharma, Shanghai, China; Akt1 siRNA: 5'-GAGUUUG AGUACCUGAAGCUG-3'; negative control: 5'-UUCUCCGAACGU GUCACGUTT-3') were transfected using Lipofectamine 2000. The siRNA used for SKP2 was a 21-bp synthetic molecule corresponding to nt847–867 of the SKP2 coding region. A 21-nt siRNA duplex corresponding to the firefly luciferase gene was used as control.

Quantitative reverse-transcriptase-PCR

Complementary DNA was synthesized using PrimeScript RT-PCR Kit (TAKARA, Dalian, China). Specific primers for quantitative reverse-transcriptase-PCR are listed in Supplementary Table S1 online. The mRNA expression of target gene versus glyceraldehyde-3-phosphate dehydrogenase (internal control) was calculated by $\Delta\Delta C_t$ method.

Antibodies and western blotting

Western blot analyses were carried out using the following primary antibodies: anti-c-Myc (sc-764, 1:1,000) and anti-SKP2 (sc-7164, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). Anti-phospho-pGSK3 β (Ser9) (9336, 1:1,000), anti-phospho-Akt (Ser473) (4051, 1:1,000), and anti-Akt (9272, 1:1,000) were from Cell Signaling Technology (Beverly, MA). E-cadherin (MAB1838, 1:1,000) and N-cadherin (MAB13881, 1:1,000) were from BD Bioscience (San Jose, CA). Anti-human β -actin (1:5,000) was purchased from Sigma (St Louis, MO).

Immunohistochemistry

Tissue microarrays were obtained from US Biomax (Rockville, MD). Additional human samples were collected at the Second Affiliated Hospital of Xi'an Jiaotong University (2005–2010), with the approval by the ethics committee of the Xi'an Jiaotong University. For immunohistochemical analysis, the endogenous peroxidase activity was blocked using 3% H₂O₂ for 12 hours, followed by incubation with 5% normal goat serum and then primary antibody at 4°C overnight. Immunohistochemistry was scored as the following by three investigators: negative, 1; minimal, 2; moderate, 3; strong, 4; or maximal, 5; *P* values were calculated using *t*-test. Statistical significance was set at **P*<0.05 and ***P*<0.01. The correlation between c-Myc and SKP2 was analyzed using analysis of variance test.

Luciferase reporter assays

The pGL4-SKP2 reporter, M1, M2, M3, or M4 each were co-transfected with control vector, c-Myc plasmid, c-Myc shRNA, Akt1 siRNA, or negative control siRNA, respectively, each with pRL-SV40. Dual luciferase activities were measured 48 hours later using the dual luciferase reporter assay system (Promega, Madison, WI). For TGF- β 1 or c-Myc inhibitor treatment, pGL4-SKP2 and pRL-SV40 were transfected into cells. TGF- β 1 or c-Myc inhibitor was added 24 hours later, and the cells were incubated for additional 24 hours, and dual luciferase activities were then measured. Relative luciferase activities were expressed as means \pm SD from at least three independent experiments.

Site-directed mutagenesis

The primers encoding the SKP2 promoter c-Myc-binding site (E-box1/2/3/4), with TTAA substituted for E-box (M1/2/3/4 mutation underlined). PCR was performed with LA Taq polymerase (TAKARA) using

the pGL4-SKP2 as a template. Mutated plasmids were sequenced to verify incorporation of the E-box site mutation.

Chromatin immunoprecipitation ChIP

The ChIP analysis was performed using the ChIP Assay kit (Upstate Biotechnology, Charlottesville, VA). 10⁷ cells were crosslinked with 1% formaldehyde for 10 minutes at 37°C and then washed, lysed, and sonicated to generate 200–500 bp chromatin fragments. The samples were precleared with 60 μ l of salmon sperm DNA–protein A-agarose and subsequently incubated at 4°C overnight with 2 μ g c-Myc antibody and rabbit IgG as control. Immunocomplexes were recovered, washed thoroughly, and eluted with the ChIP elution buffer. Following the reversal of crosslinks at 65°C for 4 hours, samples were extracted with phenol/chloroform, precipitated with ethanol, and then used as templates for PCR amplification. The qPCR assay result was expressed as fold enrichment over a non-E-box-containing fragment upstream of the 6-phosphofructo-2-kinase/fructose -2,6-biphosphatase (PFKFB3) promoter (Supplementary Table S2 online) (Sans *et al.*, 2006; Stoltzman *et al.*, 2008).

Statistics

Statistical analyses were performed using two-sample, two-tailed, equal variance Student's *t*-test. Statistical significance was set at **P*<0.05 and ***P*<0.01.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Key Program 30930105, 81071299), and by projects 985 and 211 of Xi'an Jiaotong University, and was partially supported by a Faculty Research Award (University of California School of Medicine) to FL and NCET and Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT:1171) to YZ. We thank Xiaolin Zi (Department of Urology, University of California) for providing the original SKP2 promoter construct.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Acloque H, Adams MS, Fishwick K *et al.* (2009) Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* 119:1438–49
- Assoian RK, Yung Y (2008) A reciprocal relationship between Rb and Skp2: implications for restriction point control, signal transduction to the cell cycle and cancer. *Cell Cycle* 7:24–7
- Bakin AV, Tomlinson AK, Bhowmick NA *et al.* (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 275:36803–10
- Bhatt KV, Hu R, Spofford LS *et al.* (2007) Mutant B-RAF signaling and cyclin D1 regulate Cks1/S-phase kinase-associated protein 2-mediated degradation of p27Kip1 in human melanoma cells. *Oncogene* 26: 1056–66
- Bornstein G, Bloom J, Sitry-Shevah D *et al.* (2003) Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *J Biol Chem* 278:25752–7
- Bretones G, Acosta JC, Caraballo JM *et al.* (2011) SKP2 oncogene is a direct MYC target gene and MYC down-regulates p27(KIP1) through SKP2 in human leukemia cells. *J Biol Chem* 286:9815–25

- Cross DA, Alessi DR, Cohen P *et al.* (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785–9
- Dhawan P, Singh AB, Ellis DL *et al.* (2002) Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor- κ B and tumor progression. *Cancer Res* 62:7335–42
- Govindarajan B, Sligh JE, Vincent BJ *et al.* (2007) Overexpression of Akt converts radial growth melanoma to vertical growth melanoma. *J Clin Invest* 117:719–29
- Grover R, Ross DA, Richman PI *et al.* (1996) C-myc oncogene expression in human melanoma and its relationship with tumour antigenicity. *Eur J Surg Oncol* 22:342–6
- Houghton AN, Polsky D (2002) Focus on melanoma. *Cancer Cell* 2:275–8
- Huang MJ, Cheng YC, Liu CR *et al.* (2006) A small-molecule c-Myc inhibitor, 10058-F4, induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia. *Exp Hematol* 34:1480–9
- Inuzuka H, Gao D, Finley LW *et al.* (2012) Acetylation-dependent regulation of skp2 function. *Cell* 150:179–93
- Irie HY, Pearline RV, Grueneberg D *et al.* (2005) Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. *J Cell Biol* 171:1023–34
- Javelaud D, Alexaki VI, Mauviel A (2008) Transforming growth factor-beta in cutaneous melanoma. *Pigment Cell Melanoma Res* 21:123–32
- Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. *J Clin Invest* 119:1420–8
- Kamura T, Hara T, Kotoshiba S *et al.* (2003) Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation. *Proc Natl Acad Sci USA* 100:10231–6
- Katagiri Y, Hozumi Y, Kondo S (2006) Knockdown of Skp2 by siRNA inhibits melanoma cell growth *in vitro* and *in vivo*. *J Dermatol Sci* 42:215–24
- Kim SY, Herbst A, Workowski KA *et al.* (2003) Skp2 regulates Myc protein stability and activity. *Mol Cell* 11:1177–88
- Larue L, Bellacosa A (2005) Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene* 24:7443–54
- Li JQ, Wu F, Masaki T *et al.* (2004a) Correlation of Skp2 with carcinogenesis, invasion, metastasis, and prognosis in colorectal tumors. *Int J Oncol* 25:87–95
- Li Q, Murphy M, Ross J *et al.* (2004b) Skp2 and p27kip1 expression in melanocytic nevi and melanoma: an inverse relationship. *J Cutan Pathol* 31:633–42
- Lin CP, Liu JD, Chow JM *et al.* (2007) Small-molecule c-Myc inhibitor, 10058-F4, inhibits proliferation, downregulates human telomerase reverse transcriptase and enhances chemosensitivity in human hepatocellular carcinoma cells. *Anti-Cancer Drugs* 18:161–70
- Nakayama KI, Nakayama K (2005) Regulation of the cell cycle by SCF-type ubiquitin ligases. *Semin Cell Dev Biol* 16:323–33
- Popov N, Wanzel M, Madiredjo M *et al.* (2007) The ubiquitin-specific protease USP28 is required for MYC stability. *Nat Cell Biol* 9:765–74
- Qiao D, Meyer K, Friedl A (2012) Glypican-1 stimulates Skp2 autoinduction loop and G1/S transition in endothelial cells. *J Biol Chem* 287:5898–909
- Reichert M, Saur D, Hamacher R *et al.* (2007) Phosphoinositide-3-kinase signaling controls S-phase kinase-associated protein 2 transcription via E2F1 in pancreatic ductal adenocarcinoma cells. *Cancer Res* 67:4149–56
- Robertson GP (2005) Functional and therapeutic significance of Akt deregulation in malignant melanoma. *Cancer Metastasis Rev* 24:273–85
- Rose AE, Wang G, Hanniford D *et al.* (2011) Clinical relevance of SKP2 alterations in metastatic melanoma. *Pigment Cell Melanoma Res* 24:197–206
- Sans CL, Satterwhite DJ, Stoltzman CA *et al.* (2006) MondoA-Mlx heterodimers are candidate sensors of cellular energy status: mitochondrial localization and direct regulation of glycolysis. *Mol Cell Biol* 26:4863–71
- Schlagbauer-Wadl H, Griffioen M, van Elsas A *et al.* (1999) Influence of increased c-Myc expression on the growth characteristics of human melanoma. *J Invest Dermatol* 112:332–6
- Shen L, Liu X, Hou W *et al.* (2010) NDRG2 is highly expressed in pancreatic beta cells and involved in protection against lipotoxicity. *Cell Mol Life Sci* 67:1371–81
- Smith AP, Verrecchia A, Faga G *et al.* (2009) A positive role for Myc in TGF beta-induced Snail transcription and epithelial-to-mesenchymal transition. *Oncogene* 28:422–30
- Stoltzman CA, Peterson CW, Breen KT *et al.* (2008) Glucose sensing by MondoA: Mlx complexes: a role for hexokinases and direct regulation of thioredoxin-interacting protein expression. *Proc Natl Acad Sci USA* 105:6912–7
- Takahashi K, Tanabe K, Ohnuki M *et al.* (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–72
- Tang Y, Simoneau AR, Liao WX *et al.* (2009) WIF1, a Wnt pathway inhibitor, regulates SKP2 and c-myc expression leading to G1 arrest and growth inhibition of human invasive urinary bladder cancer cells. *Mol Cancer Ther* 8:458–68
- Totary-Jain H, Sanoudou D, Dautriche CN *et al.* (2012) Rapamycin resistance is linked to defective regulation of Skp2. *Cancer Res* 72:1836–43
- Tsvetkov LM, Yeh KH, Lee SJ *et al.* (1999) p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* 9:661–4
- Wilkes MC, Mitchell H, Penheiter SG *et al.* (2005) Transforming growth factor-beta activation of phosphatidylinositol 3-kinase is independent of Smad2 and Smad3 and regulates fibroblast responses via p21-activated kinase-2. *Cancer Res* 65:10431–40
- Yokoi S, Yasui K, Mori M *et al.* (2004) Amplification and overexpression of SKP2 are associated with metastasis of non-small-cell lung cancers to lymph nodes. *Am J Pathol* 165:175–80
- Zavadil J, Bottinger EP (2005) TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* 24:5764–74
- Zeisberg M, Neilson EG (2009) Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest* 119:1429–37